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14. ABSTRACT

Cancer stem cells (CSCs), a cell population with acquired perpetuating self-renewal properties which resemble normal stem cells, specifically in the ability to infinitely give rise to the bulk of a tumor as the "seed" of the cancer, account for cancer initiation, progression, recurrence, and chemo-resistance. The cell polarity machinery has been strongly suspected of playing an evolutionarily-conserved role in regulating the cell fate in both normal and neoplastic stem cell populations, which suggests that therapeutic targeting of this mechanism may be an effective strategy for eliminating CSCs and thereby impeding cancer progression and recurrence. During the first grant period, we have successfully completed the proposed studies in Aim1 and have also made significant progress in the ongoing experiments in Aim2. Our results collectively support the hypothesis that PKCzeta is a novel target of microRNA-200c (miR-200c), the most significantly down-regulated miRNA in breast CSCs. Dysregulation of miR200c-PKCzeta signaling is critical for sustaining a self-renewing breast CSC pool and is associated with high-grade aggressive breast cancer. Together, these data point to a great potential for the strategies targeting PKCzeta signaling to exhaust the CSC pool for treatment of breast cancer.

15. SUBJECT TERMS
Breast cancer, PKCzeta, MicroRNA, Cancer stem cell, Cell fate determinant, Cell polarity

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1. Introduction

Cancer stem cells (CSCs), a cell population with acquired perpetuating self-renewal properties which resemble normal stem cells, specifically in the ability to infinitely give rise to the bulk of a tumor as the "seed" of the cancer, account for cancer initiation, progression, recurrence, and chemo-resistance. To date, treatment strategies designed to eliminate CSCs still remain a significant challenge, and delineation of the underlying mechanism(s) governing the cell fate decision to maintain self-renewal properties in CSCs likely holds the key to the development of effective treatments that can eradicate the genesis of cancer. The cell polarity machinery has been strongly implied to play an evolutionarily-conserved role in regulating cell fate in both normal stem cells and cancer stem cells (CSCs), suggesting that therapeutic targeting of this mechanism may be an effective strategy that can be applied to eliminate CSCs and thereby to impede cancer progression, recurrence, and chemo-resistance. However, the precise critical cell polarity components and mechanisms involved in the regulation of CSC cell fate still remain to be defined. Notably, asymmetric divisions (AD) is a critical mechanism which ensures self-renewal during proliferation of mammalian stem cells, where a family of cell polarity proteins, atypical Protein Kinase C (aPKC), phosphorylates the cell fate determinant NUMB, which in turn directs the polarized distribution of NUMB exclusively to the daughter cell with the differentiated cell fate, allowing the opposite daughter cell that accumulates aPKC to maintain the stem cell identity. In contrast, loss of PKCζ expression/activity leads to a uniform distribution of NUMB with the consequent symmetric commitment (SC) of both daughter cells to the differentiated cell fate, resulting in exhaustion of the stem cell pool. Interestingly, our preliminary data provide the first evidence showing that a member of the aPKC family, PKCζ, is a novel target of microRNA-200c (miR-200c), a microRNA known to be significantly down-regulated in breast CSCs. Our own previous findings and the preliminary results in this study further elucidate that loss of miR-200c not only leads to the gain of stem cell properties to generate a CSC-like population, but also enhances AD to sustain the CSC pool, potentially through upregulation of PKCζ. Even though these findings provide evidence to support a role of miR200c-PKCζ axis in regulation of breast CSCs, the precise underlying mechanism that links the regulation of PKCζ to the breast CSC fate remain to be delineated, and the analysis system to elucidate the dynamic changes of the CSC fate decision (AD vs. SC) has yet to be established. As a consequence, there remains a critical need to determine the mechanisms by which the CSC fate is regulated, since, in the absence of such knowledge, the development of effective therapeutic interventions to target CSCs and prevent cancer progression and recurrence will likely remain limited. Based on supporting evidence and our own preliminary data, our central hypothesis is that upregulation of PKCζ expression is critical for promoting AD to sustain a self-renewing CSC pool, and that strategies targeting PKCζ signaling will be therapeutically effective in treating breast cancer by exhausting CSCs. To test the hypothesis, we propose the following aims: Aim 1 will determine the key cell polarity mechanism(s) involved in regulation of breast CSCs, and Aim 2 will develop a therapeutic strategy targeting the cell polarity machinery to direct breast CSC fate. At the completion of this project, it is our expectation that we will have revealed a novel role of miR-200c- PKCζ signaling in regulation of the polarity of breast CSC division and the consequent cell fate, and have provided new and important clinical implication of PKCζ inhibitor in breast cancer treatment. Under the support of this award, we have made the following progress during the **first** grant period (Sep 30, 2015- Oct 1, 2016).

2. Keywords

Breast cancer, PKCzeta, MicroRNA, Cancer stem cell, Cell fate determinant, Cell polarity

3. Accomplishments

- **Goals and Accomplishments:**

Specific Aim 1: Determine the key cell polarity mechanism(s) involved in regulation of breast CSCs

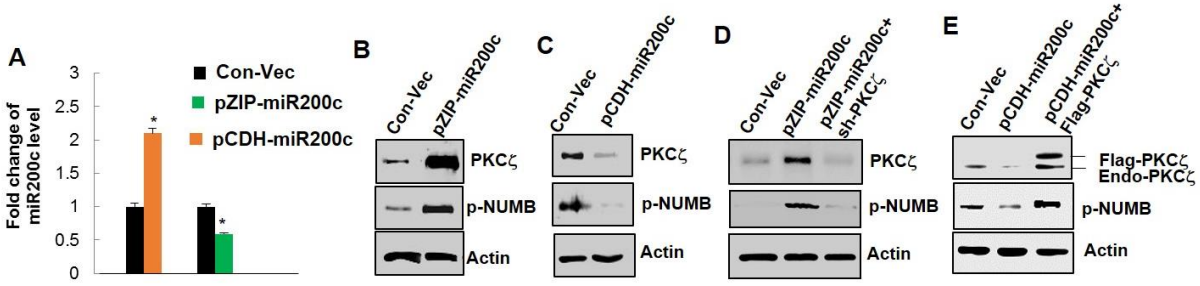


Figure 1. PKCζ is a bona fide miR-200c target. (A) MiR-200c expression levels (n=3 independent experiments, asterisk indicates P<0.05, error bars denote ±SD), and **(B-C)** PKCζ and p-NUMB protein levels in MCF7-pZIP-miR200c cells that stably expressed sh-miR200c, in BT549-pCDH-miR200c cells that stably expressed miR-200c, and in the control cells expressing the control vectors. **(D-E)** PKCζ and p-NUMB protein levels in MCF7-pZIP-miR200c cells that further expressed sh-PKCζ, in BT549-pCDH-miR200c cells that further expressed PKCζ cDNA, and in the control cells expressing the control vectors.

<u>Major Task 1: Determine the role of miR200c-PKCζ signaling in regulation of breast CSCs (Months 1-8)</u>

Subtask 1: Establish BT549 cells that stably express miR-200c (pCDH-miR200c) and MCF7 cells with knock-down of miR-200c (pZIP-miR200c) (Months 1-2) **<u>Completed!</u>**

We have successfully established BT549-pCDH-miR200c and MCF7-pZIP-miR200c and examined the protein expression levels as described in subtask 2 (Fig. 1).

Subtask 2: Determine expression levels of PKCζ and phospho-NUMB (p-NUMB), by re-expressing PKCζ in BT549-pCDH-miR200c cells and knocking-down PKCζ in MCF7-pZIP-miR200c cells (Months 3-5) **<u>Completed!</u>**

We have successfully demonstrated that PKCζ is a bona fide miR-200c target. PKCζ protein expression and the phosphorylation level of its substrate p-NUMB are markedly

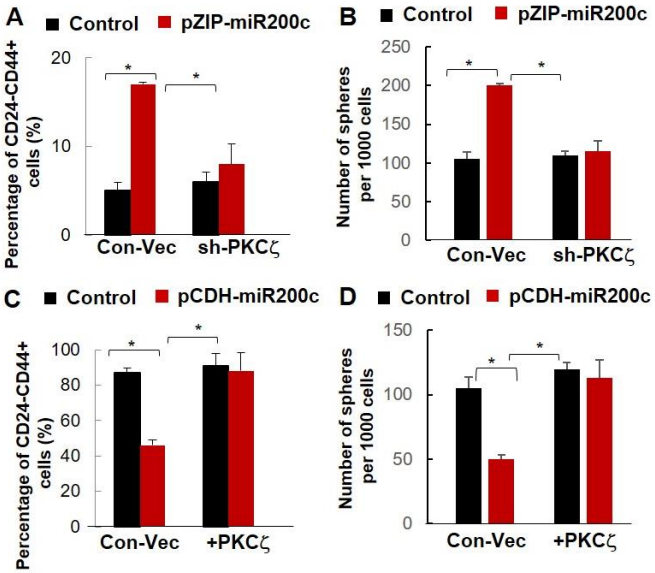


Figure 2. MiR-200c suppresses breast CSC traits through down-regulation of PKCζ. (A) The percentage of isolated $CD24^{-}CD44^{+}$ population, and **(B)** the number of tumor spheres (sphere size>100μm) per 1000 initially plated cells from MCF7-pZIP-miR200c cells that further expressed sh-PKCζ. **(C)** The percentage of isolated $CD24^{-}CD44^{+}$ population and **(D)** the number of tumor spheres (sphere size>100μm) per 1000 initially plated cells from BT549-pCDH-miR200c cells that further expressed PKCζ. n=3 independent experiments, asterisk indicates P<0.05. Error bars denote ±SD.

upregulated by knock-down of miR-200c in MCF7-pZIP-miR200c cells, which can be reversed by knock-down of PKCζ. Consistently, PKCζ protein expression and the phosphorylation level of its substrate p-NUMB are downregulated by ectopic expression of miR-200c in BT549-pCDH-miR200c cells, which can be rescued by re-expression of PKCζ (Fig. 1).

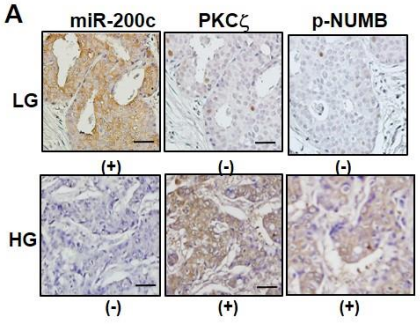
Subtask 3: Determine changes in the percentage of CD24-CD44+ cells and the sphere forming capacity by ectopically expressing PKCζ in BT549-pCDH-miR200c cells and knocking-down PKCζ in MCF7-pZIP-miR200c cells (Months 6-8) **Completed!**

We have successfully demonstrated that the percentage of $CD24^{-}CD44^{+}$ cells and the sphere forming capacity are markedly enhanced by knock-down of miR-200c in MCF7-pZIP-miR200c cells, which can be reversed by knock-down of PKCζ. Consistently, the percentage of $CD24^{-}CD44^{+}$ cells and the sphere forming capacity are suppressed by ectopic expression of miR-200c in BT549-pCDH-miR200c cells, which can be rescued by re-expression of PKCζ (Fig. 2).

Major Task 2: Determine the correlation of miR200c-PKCζ regulation with clinicopathological characteristics in human breast tissue samples (Months 9-14)

Subtask 1: Determine the expression levels of miR-200c, PKCζ, p-NUMB by in situ hybridization or immunohistochemical staining in 150 human breast tumor samples (consecutive FFPE tissue sections) collected from the Susan G. Komen for the Cure® Tissue Bank at Indiana University (Months 9-12) **Completed!**

We have performed a correlation analysis of PKCζ, p-NUMB, and miR-200c expression levels in human breast tissue specimens consisting of a cohort of breast tumor samples. We have successfully demonstrated that PKCζ and p-NUMB levels are repressed in the well-differentiated low tumor grade breast tumors (LG, grade I), where miR-200c was highly expressed (Fig. 3, n=98). In contrast, the poorly-differentiated high tumor grade tumors (HG, grade II-III) exhibit overexpression of PKCζ and p-NUMB, along with significantly reduced miR-200c levels ((Fig. 3, n=98).



B

Tumor Grade	LG	HG	
miR200c+	15 (15%)	10 (10%)	
miR200c-	11 (12%)	62 (63%)	
Total	26 (27%)	72 (73%)	*P=0.00001*
PKCζ+	7 (7%)	41 (42%)	
PKCζ-	19 (19%)	31 (32%)	
Total	26 (26%)	72 (74%)	*P=0.008*
pNUMB+	10 (10%)	42 (42%)	
pNUMB-	16 (17%)	30 (31%)	
Total	26 (27%)	72 (73%)	*P=0.01*

Figure 3. Lost miR200c expression is correlated with overexpression of PKCζ and p-NUMB in high-grade, aggressive breast cancer. (A) Representative IHC staining images showing expression levels of miR-200c, PKCζ and p-NUMB in 98 human breast tissue specimens, including low-grade tumors (tumor grade I, LG) and high-grade tumors (tumor grade II-III, HG, scale bar: 100μm). **(B)** Correlation of miR-200c, PKCζ and p-NUMB expression levels with tumor grade was analyzed by Chi-Square analysis. (-): negative-low staining, (+): strong-positive staining.

Subtask 2: Correlation analysis of the protein levels among miR-200c, PKCζ, p-NUMB and their correlation with tumor subtype (p53, BRCA1, ER/PR/HER2 status), tumor grade (differentiation status), tumor stage (metastasis status), and 5 year recurrence status (Months 13-14) **Ongoing**

We are continuously working with Dr. Weng on correlation analysis of miR-200c, PKCζ, p-NUMB expression with tumor subtype (p53, BRCA1, ER/PR/HER2 status), tumor stage (metastasis status), and 5 year recurrence status.

Specific Aim 2: Develop a therapeutic strategy targeting the cell polarity machinery to direct breast CSC fate

Major Task 3: Determine the role of miR200c-PKCζ signaling in regulation of the polarity of breast CSC division (Months 15-20)

Subtask 1: Confocal fluorescence paired cell imaging of GLI1 reporter activity, CD44, and NUMB will be analyzed during the first division of the breast CSCs expressing the control vector, miR-200c, and miR-200c+PKCζ expression plasmids or under the treatment of vehicle/PKCζ inhibitor (Months 15-17) **Ongoing**

We have established the paired cell imaging using GLI1 reporter activity, CD44, and NUMB in control CSCs (Fig. 4), which will further be analyzed in the breast CSCs expressing miR-200c, and miR-200c+PKCζ expression plasmids or under the treatment of vehicle/PKCζ inhibitor.

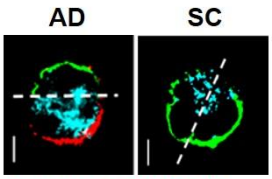


Figure 4. Representative images showing intracellular distribution of CD44 (red), NUMB (green), and GLI1-CFP reporter (aquamarine) in the dividing CSCs during asymmetric cell division (AD) or symmetric lineage commitment of the $CD24^{-}CD44^{high}$ population that was isolated from BT549 cells cultured in suspension with the 25mM blebbistatin treatment for 48 hrs (scale bar: 10μm).

Subtask 2: Serial sphere formation assay with repeated dissociation of spheres into single cells followed by re-formation of spheres for three consecutive passages using breast CSCs expressing the control vector, miR-200c, and miR-200c+PKCζ expression plasmids or under the treatment of vehicle/PKCζ inhibitor(Months 18-20) **Ongoing**

We have established the sphere cultures expressing control vector, miR-200c, and miR-200c+PKCζ plasmids, which will further be used to test the optimal concentration of PKCζ inhibitor treatment. The number of spheres per 1000 plated cells will be analyzed from three independent experiments.

Major Task 4: Determine the therapeutic effect of targeting miR200c-PKCζ signaling in vivo

Subtask 1: Determine the expected tumorigenic CSC frequency of primary breast CSCs stably expressing miR-200c, miR-200c+ PKCζ, and the control plasmids in mammary tumor xenograft animals using Extreme Limiting Dilution Analysis (Months 21-28) **Not started**

Major Task 5: Identify new lead compounds that impact the polarity of breast CSC division using phenotypic screening

Subtask 1: High throughput phenotypic screening of >200k compounds to identify the ones that direct primary breast CSCs to symmetric commitment (SC) by high content paired cell imaging analysis (Months 29-36) **Not started**

- **Opportunities for training and professional development:**
  1. The award provides training opportunities to the postdoc fellow, Yu-Syuan Chen, and the graduate student, Meng-Ju Wu, to receive courses, mentoring, and research experience that have advanced their professional skills.

  2. The award provides professional development opportunities to the graduate student, Meng-Ju Wu, for participation in conferences, such as 2016 American Association of Cancer Research Annual Conference.

- **Results disseminated to communities of interest:**
  Nothing to report

- **Plan to do during the next reporting period to accomplish the goals:**
  We have successfully completed the proposed studies in Aim1 with the results that are highly supportive of our central hypothesis. We will continue determining the therapeutic effect of a specific PKCζ inhibitor on the CSC fate and CSC frequency in vitro and in the breast cancer xenograft animals as planned in the major task 3 and 4 during the next reporting period.

4. Impact

The impact on the development of the principal discipline(s) of the project:

About 1 in 8 U.S. women will develop breast cancer over the course of her lifetime, and in the year of 2015, breast cancer has claimed the lives of approximately 40,000 women and men in the United States. Although initial remission can be achieved with chemo-treatments, the worry and fear of treatment resistance, recurrence, and death still have a deep impact on many breast cancer patients. It is recognized that cancer stem cells (CSCs), a long-lived, self-perpetuating cell population that can infinitely give rise to the bulk of a tumor as the "seed" of the cancer, account for cancer initiation, progression, radio-/chemo-resistance, and recurrence. To date, treatment strategies designed to eliminate the genesis of the cancer (CSC) still remain a significant challenge. This project aims to identify critical cell components and their working mechanisms that are used to sustain the breast CSC pool, and the identified mechanism will further be therapeutically targeted to direct CSCs to a terminally dormant cell fate and become sensitive towards radio-/chemo-therapy. With the common properties of CSCs between many cancer types, we believe that the applications generated from our research will continuingly contribute to overcoming the therapeutic hurdles of a broad spectrum of cancers and significantly benefit the cancer patient and the survivor community for decades.

The impact on other disciplines:

Nothing to report.

The impact on technology transfer:

Nothing to report.

The impact on society beyond science and technology:

Nothing to report.

5. Changes/Problems

Nothing to report

6. Products

- **Publications, conference papers, and presentations:**

(1) Journal publications (#: corresponding author):

a. Wu MJ, Chen YS, and Chang C-J[#] (2016) Retinoic acid directs stem cell fate through regulation of TET2-PKCζ pathway in breast cancer. Oncogene (Accepted, acknowledgement of federal support-yes)

b. Kim M, Wu MJ, and Chang C-J[#] (2016). Role of microRNA in regulation of mammary stem cell fate and tumorigenesis. Stem Cells International (Re-submitted with Revisions, acknowledgement of federal support-yes)

(2) Presentations:

a. Keystone Symposia- Stem Cells & Cancer, Breckenridge,
"Epigenetic regulation promotes obesity related breast cancer progression"

b. Annual Meeting of International Society for Stem Cell Research, San Francisco, CA
"The role of miR200c in ATRA-mediated differentiation of breast cancer stem cells" (*)

c. Purdue University Center for Cancer Research
West Lafayette, IN
"The role of Tet2 in mammary stem cell fate and tumorigenesis"

- **Technologies, inventions, patent applications, and/or licenses:**
Nothing to reports.

- **Other Products:**
a. Establishment and validation of BT549-pCDH-miR200c cells that stably express miR-200c and MCF7-pZIP-miR200c cells that stably knock-down miR-200c. These cell lines will be used for proposed in vivo experiments.

b. Generation of ChIP-sequencing and microarray datasets (GSE85189, GSE85141) as reported in the newly-accepted Oncogene paper (6-1-b).

7. Participants & Other Collaborating Organizations

- **Individuals and other support:**
  No change (Chun-Ju Chang-PI, Yu-Syuan Chen- Postdoc, Meng-Ju Wu- Graduate student).

- **Other involved organizations:**
  Nothing to report.

8. Special Reporting Requirements

Nothing to report.

9. Appendices

The abstract of our newly-accepted manuscript "Retinoic acid directs stem cell fate through regulation of TET2-PKCζ pathway in breast cancer" in Oncogene (Oct 2016)

Abstract:
The key molecular mechanism governing the stem cell fate to control the cancer stem cell (CSC) pool remains elusive. This study provides the first evidence showing that all-trans retinoic acid (ATRA) induces the interaction and chromatin recruitment of a novel RARβ-TET2 complex to epigenetically activate a specific cohort of gene targets, including *MiR-200c*. TET2-activated miR-200c further targets and suppresses PKCζ, a cell polarity protein that plays a pivotal role in directing self-renewing asymmetric division of mammalian stem cells to sustain the stem cell pool. Our data reveals that pharmacological concentration of ATRA effectively downregulates PKCζ through activation of miR-200c, leading to exhaustion of the stem cell-like populations from non-tumorigenic mammary epithelial cells and non-aggressive breast cancer cells. However, aggressive breast cancer cells that manifest TET2-miR200c dysregulation sustain a self-renewing CSC pool highly resistant to ATRA, where inhibition of PKCζ significantly suppresses of the resistant CSCs. Together, these findings elucidate a novel TET2-miR200c-PKCζ signaling pathway that governs the stem cell fate to eradicate breast cancer.